Kidney International, Vol. 63 (2003), pp. 1249–1255

HORMONES – CYTOKINES – SIGNALING

IGF-I induces vascular endothelial growth factor in human mesangial cells via a Src-dependent mechanism[1]

Gabriella Gruden, Shawanna Araf, Silvia Zonca, Davina Burt, Stephen Thomas, Luigi Gnudi, and GianCarlo Viberti

Department of Diabetes and Endocrinology, GKT School of Medicine, King's College London, London, England, United Kingdom

IGF-I induces vascular endothelial growth factor in human mesangial cells via a Src-dependent mechanism.

Background. Both insulin-like growth factor-I (IGF-I) and vascular endothelial growth factor (VEGF) have been implicated in the pathogenesis of early renal dysfunction in diabetes. We investigated whether IGF-I affects VEGF gene expression and protein secretion in human mesangial cells. Furthermore, we studied the intracellular signaling pathway involved and the interaction of IGF-I with mechanical stretch, a known VEGF inducer.

Methods. Human mesangial cells were exposed to IGF-I in the presence and in the absence of (*1*) anti-IGF-I type I receptor antibody (αIR3) (1 μg/mL), a monoclonal antibody blocking the IGF-I type I receptor; (*2*) wortmannin (600 nmol/L), a phosphatidylinositol 3-kinase (PI3K) inhibitor; (*3*) 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), a specific Src inhibitor (10 μmol/L); and (*4*) cyclic stretch (~10% elongation).

Results. IGF-I induced a dose-dependent increase in VEGF protein levels (10^{-11} mol/L, 5%; 10^{-10} mol/L, 14%; 10^{-9} mol/L, 46%; 10^{-8} mol/L, 66%; 10^{-7} mol/L, 68%; $P < 0.001$). IGF-I–induced VEGF production rose by 6 hours with a peak at 12 hours, and declined by 24 hours (52%, 72%, and 34%, respectively; $P < 0.01$ at 12 hours). A corresponding 50% increase in VEGF mRNA levels was seen at 6 hours ($P < 0.01$). IGF-I–induced VEGF protein secretion was not affected by the addition of wortmannin (IGF-I, 76% vs. IGF-I + wortmannin, 79% increase over control; P = NS), but was abolished by αIR3 (IGF-I, 69% vs. IGF-I + αIR3, 0%; $P < 0.001$) and significantly reduced by PP2 (IGF-I, 50% vs. IGF-I + PP2, 14%; $P < 0.01$). Simultaneous exposure of human mesangial cells to both IGF-I and stretch failed to further increase VEGF production (IGF-I, 1.49 ± 0.05; stretch, 1.76 ± 0.05; and IGF-I + stretch, 1.83 ± 0.11).

Conclusion. IGF-I induces VEGF gene expression and protein secretion in human mesangial cells via a Src-dependent mechanism.

[1] **See Editorial by Cooper and Thomas, p. 1584.**

Key words: IGF-I, VEGF, mesangial cells, Src.

Received for publication May 29, 2002
and in revised form September 26, 2002
Accepted for publication November 13, 2002

Insulin-like growth factor-I (IGF-I) is a potent mitogenic polypeptide, under growth hormone regulation, which binds to a specific receptor (IGF-RI) and to six binding proteins (IGFBPs) that modulate its bioavailability [1–3].

Although circulating IGF-I levels are normal or reduced in patients with diabetes [4], the local IGF-I system appears to be up-regulated. There is increased kidney IGF-I content in experimental diabetes [2, 3, 5]. Furthermore, increases in IGF-RI and changes in IGFBPs expression, leading to enhanced IGF-I trapping and possibly bioactivity, have been reported in kidneys from diabetic animals [2, 3, 6, 7].

Transgenic animals overexpressing IGF-I develop glomerular hypertrophy [7] and IGF-I infused in pharmacologic concentrations in humans increases renal plasma flow and glomerular filtration rate [8]. Furthermore, suppression of the growth hormone/IGF-I axis prevents the onset of these early glomerular abnormalities in diabetic animals [3, 5, 10, 11].

Similarly, in experimental diabetes, inhibition of vascular endothelial growth factor (VEGF) prevents both glomerular hyperfiltration and glomerular hypertrophy and ameliorates albuminuria [12]. VEGF exists in five isoforms, $VEGF_{165}$ being the most abundant. The main VEGF binding sites are on endothelial cells, and VEGF stimulates and promotes vascular permeability and endothelium-dependent vasodilatation [13]. VEGF is expressed constitutively in the kidney in glomerular epithelial cells [14] and in pathologic conditions by other glomerular cell types, including mesangial cells [15]. Both high glucose and mechanical stretch, mimicking glomerular capillary hypertension, are potent VEGF inducers in vitro in human mesangial cells [16, 17].

Given the similarities between the glomerular effects of IGF-I and VEGF, we tested in human mesangial cells whether IGF-I stimulates VEGF gene expression and protein secretion and examined the signal transduction

mechanisms involved. We also studied the interaction of IGF-I and mechanical stretch on VEGF production.

METHODS

Materials

All materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Fetal calf serum (FCS) was obtained from Gibco-BRL (Paisley, UK) and Flex I and Flex II plates from Flexcell International Corporation (McKeensport, PA, USA). Wortmannin, monoclonal anti-IGF-I type I receptor antibody (αIR3), and 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) were obtained from Calbiochem (Nottingham, UK). Monoclonal anti-human VEGF antibody was obtained from R&D Systems (Minneapolis, MN, USA) and rabbit polyclonal antihuman VEGF antibody from Serotec (Oxford, UK). The reverse transcription (RT) system and the transforming growth factor-β1 (TGF-β1) enzyme-linked immunosorbent assay (ELISA) were purchased from Promega (Southampton, UK), oligonucleotide primers from Oswel (Southampton, UK), and AmpliTaq from Perkin Elmer (Warrington, WA, USA).

Cell culture

Human mesangial cells were isolated as described previously [17, 18]. Briefly, normal renal cortex was obtained from three donor nephrectomies found to be unsuitable for transplantation on the basis of an abnormal vascular supply. Intact glomeruli were collected from cortical homogenates by serial sieving. The isolated glomeruli were digested with collagenase (type IV, 750 U/mL) and then seeded in culture flasks. After the outgrowth of mesangial cells, the glomeruli were removed by washing and the cells were cultured in RPMI 1640 medium, supplemented with insulin-transferrin-selenium and L-glutamine, and containing 20% FCS, 7 mmol/L glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 incubator at 37°C. Mesangial cells were harvested using 0.25% trypsin and 0.5% ethylenediaminetetraacetic acid (EDTA). The cells were stellate or fusiform in appearance, grew in multilayers, formed hillocks in long-term culture, and stained for α-smooth muscle actin (α-SMA) by direct immunofluorescence. Cells did not stain for cytokeratin, factor VIII, common leukocyte antigen (DAKO, High Wycombe, UK) and Thy-1 (Serotec, Oxford, UK), excluding, respectively, contamination of epithelial, endothelial cells, lymphomonocytes, and human fibroblasts. Studies were performed between passages 4 and 7, while the cells retained the characteristic morphologic and immunofluorescent features described above.

Cell number determination

Cell were harvested with 0.25% trypsin and 0.5% EDTA and the cell number determined by a Coulter Cell Counter (Coulter Electronics, Ltd., Luton Beds, UK).

Application of mechanical stretch to cultured cells

Mesangial cells were seeded in equal number (12,000/cm^2) into six-well type I collagen-coated silicone elastomer-base culture plates (Flex I plates) and control plates (Flex II plates). After insulin and serum deprivation for 24 hours, cells were subjected to repeated stretch/relaxation cycles (60 cycles/minute) by mechanical deformation using a Flexercell Strain Unit (FX3000 Flexcell International Corporation, McKeensport, PA, USA). Cells were exposed to an average 10% uniaxial elongation, which mimics that present in vivo in glomeruli exposed to supernormal pressure levels, and is known in vitro to induce mesangial cell VEGF production [17]. Stretch and control experiments were carried out simultaneously with cells derived from a single pool. Control cells were grown in nondeformable, but otherwise identical, plates (Flex II plates) in parallel.

mRNA analysis

Total RNA was isolated using a commercial preparation based on a guanidinium and phenol extraction (Triazol) and reverse transcribed (1 μg) according to standard protocols using avian myeloblastosis virus (AMV) reverse transcriptase and poly-d(T). The polymerase chain reaction (PCR) was performed with oligonucleotide primers designed to amplify specifically the 165 isoform of human VEGF as we have previously described [17]. A single PCR product of 317 bp was obtained, the identity of which was confirmed by digestion with the restriction enzyme *Hin*dIII (Promega) yielding two fragments of 181 bp and 136 bp as predicted from the known cDNA sequence for $VEGF_{165}$ [19]. Expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined in parallel to control for amount of RNA input and RT efficiency using primer sequences previously reported [20]. VEGF and GAPDH mRNA levels were quantified by competitive RT-PCR, using deletion-mutated cDNA to control for PCR amplification efficiency and for use in quantitative analysis [17]. Competitor cDNAs with a 50 bp deletion were generated by PCR as previously described [21]. PCR products were resolved in a 3% Nu-Sieve/1% agarose gel containing ethidium bromide, analyzed by an image system (Eagle Eye System, Strategene, UK) and quantified using a densitometry analysis software (QGEL, Strategene, UK).

Protein measurement

Culture supernatants from all experimental conditions were collected, centrifuged to remove cell debris, and

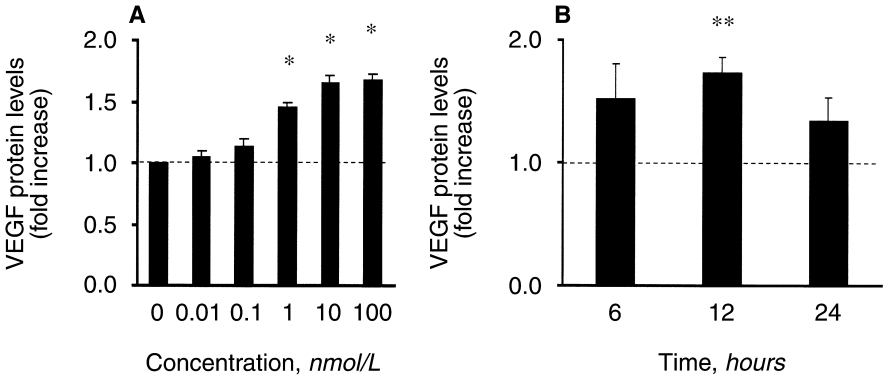

Fig. 1. Dose-response and time course of vascular endotheial growth factor (VEGF) protein production by insulin-like growth factor-I (IGF-I) in human mesangial cells. Serum- and insulin-deprived human mesangial cells were exposed to increasing IGF-I concentrations (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} mol/L) for 12 hours (*A*); and to IGF-I at 10 nmol/L for various time periods of 6, 12, and 24 hours (*B*). VEGF protein levels were measured as described in the **Methods** section and expressed as fold increase vs. control $*P < 0.001$ vs. control ($N = 3$); $**P < 0.01$ vs. control ($N = 4$).

stored at −70°C for analysis. VEGF protein concentration was measured by an in-house two-site immunoenzymometric assay using a mouse monoclonal and a rabbit polyclonal antihuman $VEGF_{165}$ (range, 1 to 40 pmol/L, intra-assay coefficient of variance, 5.3%) as we have previously reported [17, 22]. Briefly 96-well microtiter plates were coated overnight at 4°C with a mouse monoclonal anti-VEGF antibody as the capture antibody. The plates were blocked with bovine serum albumin (BSA), following which the samples were added and incubated for 5 hours. After washing, a rabbit polyclonal antihuman $VEGF_{165}$ as the detection antibody was added and incubated overnight. Immunocomplexes were detected by alkaline phosphatase-conjugated goat antirabbit immunoglobulin (IgG) and revealed by 3,3′, 5,5′ tetramethylbenzidine dihydrochloride substrate. The reaction was stopped with H_2S0_4 and the absorbance was measured at 450/690 nm. The assay also detects the $VEGF_{121}$ isoform, but no cross-reactivity was detected with human platelet-derived growth factor (PDGF), human TGF-β1 to 5 and bovine VEGF. For each experiment, VEGF protein levels were determined within a single assay run. All protein results were adjusted for cell number.

Total TGF-β1 protein concentration was measured by ELISA (range, 16 to 1000 pg/mL, intra-assay coefficient of variance, 1.6%) using a mouse monoclonal and a rabbit polyclonal antihuman TGF-β1. Activation of latent TGF-β1 was obtained by acidification according to manufacturer's instructions.

Data presentation and statistical analysis

All data are presented as mean ± SEM. Data were analyzed by analysis of variance (ANOVA) and if significant, the Newman-Keuls procedure was used for post hoc comparisons. Values for $P < 0.05$ were considered significant.

RESULTS

IGF-I induces VEGF gene expression and protein secretion

To investigate the effect of IGF-I on VEGF protein production, serum and insulin-deprived mesangial cells were exposed to increasing IGF-I concentrations (10^{-11}, 10^{-10}, 10^{-9}, 10^{8}, and 10^{-7} mol/L) for 12 hours to determine the effective IGF-I dose. Twelve hours was chosen as being a known time period in which various stimuli upregulate VEGF protein production [17, 22]. Cells were serum- and insulin-deprived for 24 hours prior to the experiment because VEGF and IGF-I receptor expression are enhanced and diminished, respectively, by small concentrations of FCS [15, 23]. IGF-I induced VEGF secretion in a concentration-dependent manner (10^{-11} mol/L, 5%; 10^{-10} mol/L, 14%; 10^{-9} mol/L, 46%; 10^{-8} mol/L, 66%; and 10^{-7} mol/L, 68%) with a minimum effective concentration of 1 nmol/L and a plateau effect at 100 nmol/L (Fig. 1A). In subsequent experiments a 10 nmol/L IGF-I concentration was used as this concentration maximally induced VEGF.

A significant increase in VEGF production was also observed in mesangial cells exposed to insulin; however, this occurred only at pharmacologic insulin concentration of 1 μmol/L (1.78 ± 0.05-fold increase over control, $P < 0.05$, $N = 3$).

The effect of IGF-I on VEGF secretion was not accompanied by an increase in TGF-β1, another growth factor produced by mesangial cells and believed to be important in the pathogenesis of diabetic glomerulopathy (IGF-I at 10 nmol/L, 1.06 ± 0.03-fold increase over control, P = NS, $N = 3$).

In time-course experiments, we observed IGF-I–induced VEGF secretion by 6 hours, with a peak at 12 hours and a decline thereafter (52%, 72%, and 34% respectively; $P < 0.01$ IGF-I over control at 12 hours) (Fig. 1B).

To assess whether this rise in VEGF protein secretion was preceded by an increase in mRNA levels, we measured by competitive RT-PCR VEGF mRNA in cells exposed to IGF-I (10 nmol/L) for 6 hours. This time point was chosen based on previous studies showing that VEGF mRNA levels rise approximately 6 hours earlier than VEGF protein levels [17, 22]. VEGF was expressed in the basal condition and significantly rose by 50% in cells exposed to IGF-I (Fig. 2).

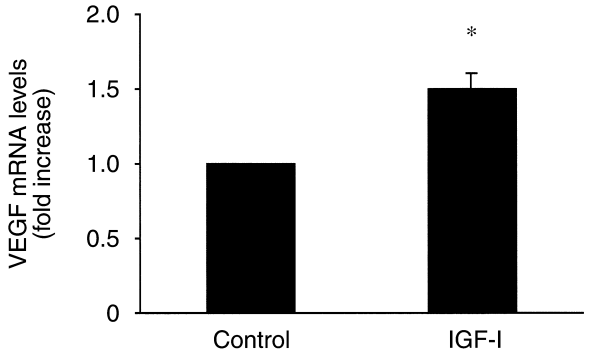

Fig. 2. Effect of insulin-like growth factor (IGF-I) on vascular endothelial growth factor (VEGF) mRNA levels. Serum and insulin-deprived human mesangial cells were exposed to IGF-I (10 nmol/L) for 6 hours. VEGF mRNA levels were quantified as described in the **Methods** section and expressed as fold increase vs. control. * $P < 0.01$ IGF-I over control ($N = 4$).

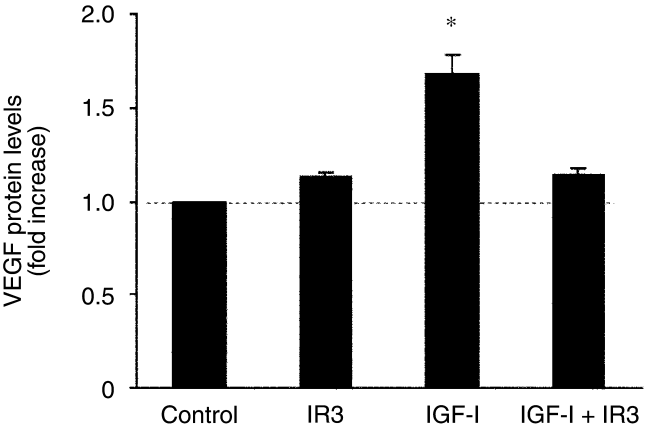

Fig. 3. Insulin-like growth factor-I (IGF-I) induces vascular endothelial growth factor (VEGF) via the IGF-I type 1 receptor. Serum- and insulin-deprived human mesangial cells were exposed to IGF-I (10 nmol/L) for 12 hours in the presence or in the absence of αIR3 (IR3 1 μg/mL). VEGF protein levels were measured as described in the **Methods** section and expressed as fold increase vs. control. *$P < 0.001$ IGF-I vs. others ($N = 3$).

Mesangial cell VEGF induction by IGF-I occurs via the IGF-RI receptor

IGF-I binds to the IGF-RI receptor as well as to the insulin receptor, although with a 50- to 100-fold reduction in affinity [24]. A murine monoclonal antibody against the IGF-RI, αIR3 (1 μg/mL), specifically recognizes the extracellular α-subunit of the IGF-RI and inhibits receptor-mediated effects [25] Preincubation with αIR3 (1 μg/mL) for 30 minutes before IGF-I (10 nmol/L) addition led to complete inhibition of IGF-I–induced VEGF protein production (Fig. 3), indicating that this effect was mediated exclusively by the IGF-RI receptor. The αIR3 neutralizing antibody induced a modest and not significant rise in basal VEGF levels due to its weak agonist activity [26].

IGF-I–induced VEGF secretion is independent of PI3K

To test the role of phosphatidylinositol 3-kinase (PI3K), an intracellular mediator of IGF-I signaling [27, 28], in IGF-I–induced VEGF production, serum- and insulin-deprived mesangial cells were exposed to IGF-I (10 nmol/L) for 12 hours either in the presence or in the absence of the PI3K inhibitor, wortmannin (600 nmol/L). IGF-I–induced VEGF secretion was not affected by the addition of wortmannin, indicating that VEGF induction was PI3K-independent (Fig. 4A). By contrast, in parallel experiments, wortmannin completely abolished insulin-induced VEGF production [insulin (1 μmol/L) + vehicle, 1.78 ± 0.03; insulin (1 μmol/L) + wortmannin (600 nmol/L), 1.12 ± 0.04-fold increase over control; $P < 0.05$ insulin vs. others, $N = 3$]. This indicates that insulin induces VEGF through a PI3K-dependent pathway, and it confirms that, in our experiments, wortmannin was both active in the preparation and used at a concentration adequate to block PI3K-dependent effects.

Inhibition of Src by PP2 prevents IGF-I–induced VEGF secretion

Src is known to mediate stretch-induced VEGF in human mesangial cells [17]. Thus, we tested whether this intracellular signaling molecule could also be involved in IGF-I–induced VEGF. Serum- and insulin-deprived human mesangial cells were exposed to IGF-I (10 nmol/L) for 12 hours, either in the presence or in the absence of PP2 (10 μmol/L), a selective Src inhibitor [29]. PP2 induced a modest nonsignificant reduction in basal VEGF production, but significantly reduced, by 72%, IGF-I–induced VEGF secretion ($P < 0.01$), providing evidence of a Src role in IGF-I induction by VEGF (Fig. 4B).

Simultaneous exposure to IGF-I and stretch does not enhance VEGF production

To study the effect of combined exposure to IGF-I and stretch on human mesangial cell VEGF production, serum- and insulin-deprived mesangial cells were stretched (elongation 10%), either in the presence or in the absence of IGF-I (10 nmol/L) for 12 hours. Both IGF-I and stretch induced a significant increase in VEGF production; however, simultaneous exposure to both stimuli failed to further increase VEGF production (Fig. 5).

DISCUSSION

This work demonstrates that IGF-I induces VEGF production in human mesangial cells. There was a peak 72% increase after 12 hours. This increase in VEGF is commensurate with that we have previously reported in response to angiotensin II in human mesangial cells [22]. The doses of IGF-I eliciting VEGF production were in

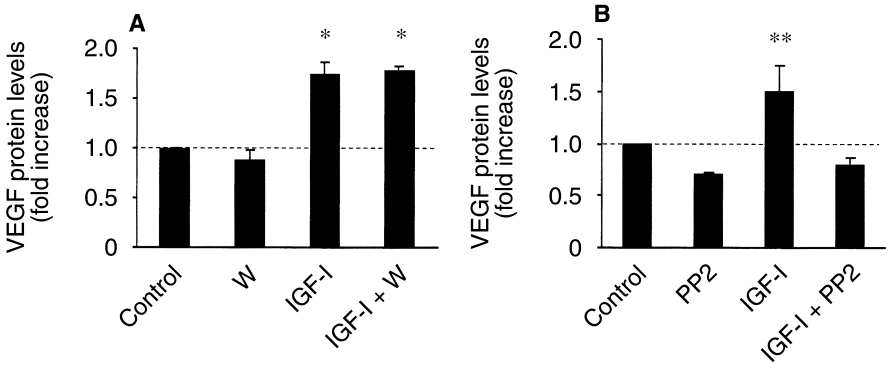

Fig. 4. Effect of phosphatidylinositol 3-kinase (PI3K) and Src inhibition on insulin-like growth factor-I (IGF-I)–induced vascular endothelial growth factor (VEGF) production in human mesangial cells. Serum- and insulin-deprived human mesangial cells were exposed to IGF-I (10 nmol/L) for 12 hours in the presence or in the absence of: wortmannin (600 nmol/L) (*A*), and 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine (PP2) (10 μmol/L) (*B*). VEGF protein levels were measured as described in the **Methods** section and expressed as fold increase vs. control. $*P < 0.001$ IGF-I and IGF-I + wortmannin vs. others ($N = 3$); $**P < 0.01$ IGF-I vs. others ($N = 3$).

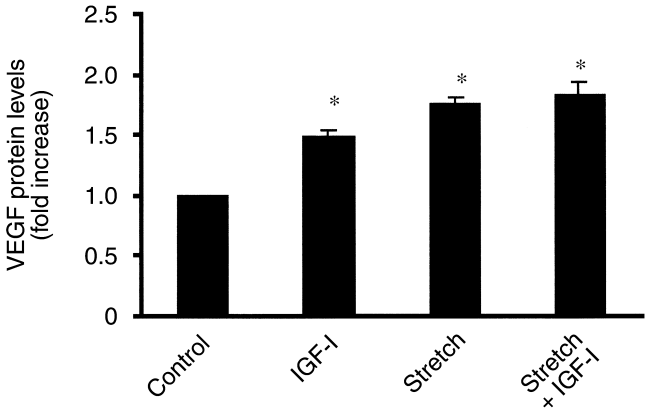

Fig. 5. Effect of combined insulin-like growth factor (IGF-I) and stretch on vascular endothelial growth factor (VEGF) protein secretion. Serum- and insulin-deprived mesangial cells were exposed for 12 hours to vehicle, IGF-I (10 nmol/L), stretch (10% elongation), and IGF-I + stretch. VEGF protein levels were measured as described in the **Methods** section and expressed as fold increase vs. control ($N = 3$). $*P < 0.05$ IGF-I, stretch, and IGF-I + stretch over control. $P =$ NS IGF-I vs. stretch vs. stretch + IGF-I.

the physiologic range and comparable to those used in previous reports on IGF-I–induced fibronectin and collagen production in murine mesangial cells [30]. Addition of exogenous IGF-I, however, is superimposed to the IGF-I endogenously produced by mesangial cells [31] and thus mimics a state of IGF-I excess, suggesting that superphysiologic IGF-I levels are required for VEGF induction. IGF-I–induced VEGF protein level rose after 6 hours, peaked at 12 hours, and declined thereafter. A temporally related increase in VEGF mRNA level was seen at 6 hours. This time course of VEGF production was similar to that we previously observed for serum, angiotensin II and stretch-induced VEGF in human mesangial cells [17, 22, 32].

IGF-I–induced VEGF expression has been previously reported in nonrenal cell lines, such as SV40-transformed retinal pigment epithelial cells [33], endometrial adenocarcinoma cells [34], and COLO205 colon carcinoma cells [26]. The magnitude and duration of the IGF-I–induced VEGF production we observed in human mesangial cells was lower than that reported in these transformed nonrenal cell lines. The reasons for this are unclear. However, increased expression of IGF-I receptor and altered regulatory mechanisms in transformed cells may explain the enhanced response to IGF-I. Our findings, although smaller in magnitude, are likely to be more physiologically relevant as they were shown in primary cultures of normal human cells.

IGF-I–induced VEGF production was completely inhibited by αIR3, a neutralizing antibody blocking the IGF-RI receptor [25]. This indicates a specific IGF-I effect occurring via the IGF-RI receptor. IGF-I can also bind to the insulin receptor, but insulin receptors are expressed at a low level in mesangial cells and are not activated in response to the IGF-I concentrations used in this study [35]. Consistently, we found that pharmacologic insulin concentrations were required to induce VEGF production in this cell type.

Exposure to IGF-I did not affect mesangial cell production of TGF-β1, a cytokine implicated in the pathogenesis of diabetic glomerulopathy [3], demonstrating that, under our experimental conditions, VEGF induction was a specific response of mesangial cells to IGF-I.

VEGF induction occurs via different mechanisms, including an Src/Raf/mitogen-activating protein (MAP) kinase pathway [36] and a PI3K/protein kinase-B (PKB) pathway [37]. PI3K plays a key role in IGF-I intracellular signaling in most cell types and mediates IGF-I–induced VEGF expression in human osteoblast-like cells [38]. However, in our experiments the addition of wortmannin, a PI3K inhibitor, did not affect IGF-I–induced VEGF secretion, indicating that in human mesangial cells this IGF-I effect was PI3K independent.

A previous report in NIH3T3 fibroblasts had shown that VEGF induction by IGF-I and insulin occurs via different signaling pathways, which are, respectively, independent and dependent of PI3K [39]. In line with this, we found that in human mesangial cells insulin induces VEGF via a PI3K-dependent mechanism.

Members of the Src family of nonreceptor tyrosine kinases are important intracellular signaling molecules for the expression of VEGF [36, 40, 41]. Cell lines transfected with v-Src overexpress VEGF [36] and mechanical stretch induces VEGF via a Src-dependent mechanism in human mesangial cells [17]. In this study, the significant inhibition of IGF-I–induced VEGF expression by the highly specific Src inhibitor, PP2 [29], indicates that Src plays a key role in mediating VEGF production in response to IGF-I. This observation is in agreement with previous studies showing that the IGF-I receptor activates the Src/Raf pathway in other cell types [39, 42]. In addition, the small reduction of baseline VEGF by PP2 suggests that Src may also be important for the maintenance of basal VEGF levels.

We have previously demonstrated that stretch induces in mesangial cells the production of VEGF via a Src-dependent mechanism [17]. IGF-I and stretch may thus converge on a common Src-dependent signaling pathway leading to VEGF production. In keeping with this hypothesis, we found that the simultaneous exposure to IGF-I and stretch failed to further increase VEGF production.

These in vitro findings may have important in vivo implications. IGF-I transgenic animals develop glomerular hypertrophy [8] and IGF-I infusion induces glomerular hyperfiltration in both human and experimental diabetes [9, 43]. Selective growth hormone/IGF system antagonists, which normalize IGF-I kidney content, prevent the onset of glomerular abnormalities in diabetic animals [3, 5, 10, 11]

Mesangial cells overexpress IGF-I receptors in vivo in diabetic animals [44] and are thus a likely target for local IGF-I action. IGF-I–induced mesangial cell VEGF production is of particular relevance for diabetic nephropathy as VEGF is overexpressed in diabetic glomeruli [14] and VEGF blockade prevents early renal dysfunction in diabetic rats [12].

There is evidence that glomerular hyperfiltration in diabetes results from enhanced glomerular vessel nitric oxide (NO) formation due to increased endotheial nitric oxide synthase (eNOS) expression and activity [45–47]. VEGF potently stimulates eNOS expression and activity in cultured endothelial cells [48] and VEGF blockade, which prevents glomerular hyperfiltration in vivo, also normalizes glomerular capillary eNOS expression [12]. IGF-I also has vasoactive properties in vivo and induces renal vasodilatation via a NO-dependent mechanism, but this effect is transient and does not involve eNOS overexpression [49, 50]. It is, thus, conceivable that in a chronic condition such as diabetic nephropathy IGF-I mediates hyperfiltration through an increase in VEGF production.

Both IGF-I and VEGF are implicated in the pathogenesis of diabetic retinopathy [51, 52]. Multiple interactions between IGF-I and VEGF pathways have been described at the cellular level in the retina [53, 54], including an enhanced VEGF production in response to IGF-I by retinal epithelial cells [33]. Our results suggest a similarity between pathogenic mechanisms of vascular dysfunction in two separate beds of the microvascular circulation.

ACKNOWLEDGMENTS

This work was supported by the Diabetes UK grant RD/98/0001608. Ms. Shawana Araf was supported by a Wellcome Scholarship and Dr. S. Thomas by a JDRFI fellowship. Dr. Silvia Zonca was a visiting fellow from the Department of Clinical Pediatrics III of the University of Milan.

Reprint requests to Dr. Gabriella Gruden, M.D., Department of Diabetes and Endocrinology, 5th Floor, Thomas Guy House, Guy's Hospital, London SE1 9RT, United Kingdom.
E-mail: ggruden@hotmail.com

REFERENCES

1. LeRoith D, Adamo M, Werner H, Roberts CT Jr: Insulin-like growth factors, their binding proteins and receptors as growth regulators in normal physiology and pathological states. *Trends Endocrinol Metab* 2:134–141, 1991
2. Flyvbjerg A: Role of growth hormone, insulin-like growth factors (IGFs) and IGF-binding proteins in the renal complications of diabetes. *Kidney Int* 52 (Suppl 60):S12–S19, 1997
3. Flyvbjerg A: Putative pathophysiological role of growth factors and cytokines in experimental diabetic kidney disease. *Diabetologia* 43:1205–1223, 2000
4. Chestnut RE, Quarmby V: Evaluation of total IGF-I assay methods using samples from type I and type II diabetic patients. *J Immunol Methods* 259:11–24, 2002
5. Flyvbjerg A, Bennett WF, Rasch R, *et al*: Inhibitory effect of a growth hormone receptor antagonist (G120K-PEG) on renal enlargement, glomerular hypertrophy, and urinary albumin excretion in experimental diabetes in mice. *Diabetes* 48:377–382, 1999
6. Sugimoto H, Shikata K, Makino H, *et al*: Increased gene expression of insulin-like growth factor-I receptor in experimental diabetic rat glomeruli. *Nephron* 72:648–653, 1996
7. Landau D, Chin E, Bondy C, *et al*: Expression of insulin-like growth factor binding proteins in the rat kidney: Effects of long-term diabetes. *Endocrinology* 136:1835–1842, 1995
8. Doi T, Striker LJ, Gibson CC, *et al*: Glomerular lesions in mice transgenic for growth hormone and insulinlike growth factor-I. I. Relationship between increased glomerular size and mesangial sclerosis. *Am J Pathol* 137:541–552, 1990
9. Giordano M, DeFronzo RA: Acute effect of human recombinant insulin-like growth factor I on renal function in humans. *Nephron* 71:10–15, 1995
10. Landau D, Segev Y, Afargan M, *et al*: A novel somatostatin analogue prevents early renal complications in the non-obese diabetic mouse. *Kidney Int* 60:505–512, 2001
11. Haylor J, Hickling H, El Eter E, *et al*: JB3, an IGF-I receptor antagonist, inhibits early renal growth in diabetic and uninephrectomized rats. *J Am Soc Nephrol* 11:2027–2035, 2000
12. de Vriese AS, Tilton RG, Elger M, *et al*: Antibodies against vascular endothelial growth factor improve early renal dysfunction in experimental diabetes. *J Am Soc Nephrol* 12:993–1000, 2001
13. Ferrara N: Molecular and biological properties of vascular endothelial growth factor. *J Mol Med* 77:527–543, 1999
14. Cooper ME, Vranes D, Youssef S, *et al*: Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes. *Diabetes* 48:2229–2239, 1999
15. Iijima K, Yoshikawa N, Connolly DT, Nakamura H: Human mesangial cells and peripheral blood mononuclear cells produce vascular permeability factor. *Kidney Int* 44:959–966, 1993

16. Kim NH, Jung HH, Cha DR, *et al*: Expression of vascular endothelial growth factor in response to high glucose in rat mesangial cells. *J Endocrinol* 165:617–624, 2000
17. Gruden G, Thomas S, Burt D, *et al*: Mechanical stretch induces vascular permeability factor in human mesangial cells: Mechanisms of signal transduction. *Proc Natl Acad Sci USA* 94:12112–12116, 1997
18. Striker GE, Striker LJ: Biology of disease: Glomerular cell culture. *Lab Invest* 53:122–131, 1985
19. Tischer E, Mitchell R, Hartman T, *et al*: The human gene for vascular endothelial growth factor: Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 266:11947–11954, 1991
20. Maier JAM, Voulalas PM, Roeder D, Maciag T: Extension of the life-span of human endothelial cells by an interleukin-1 antisense oligomer. *Science* 249:1570–1574, 1990
21. Celi FS, Zenilman ME, Shuldiner AR: A rapid and versatile method to synthesize internal standards for competitive PCR. *Nucleic Acids Res* 21:1047, 1993
22. Gruden G, Thomas S, Burt D, *et al*: Interaction of angiotensin II and mechanical stretch on vascular endothelial growth factor production by human mesangial cells. *J Am Soc Nephrol* 10:730–737, 1999
23. Conti FG, Striker LJ, Lesniak MA, *et al*: Studies on binding and mitogenic effect of insulin and insulin-like growth factor I in glomerular mesangial cells. *Endocrinology* 122:2788–2795, 1998
24. Nissley P, Lopaczynski W: Insulin-like growth factor receptors. *Growth Factors* 5:29–43, 1991
25. Li YM, Schacher DH, Liu Q, *et al*: Regulation of myeloid growth and differentiation by the insulin-like growth factor I receptor. *Endocrinology* 138:362–368, 1997
26. Warren RS, Yuan H, Matli MR, *et al*: Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. *J Biol Chem* 271:29483–29488, 1996
27. Tack I, Elliot SJ, Potier M, *et al*: Autocrine activation of the IGF-I signalling pathway in mesangial cells isolated from diabetic NOD mice. *Diabetes* 51:182–188, 2002
28. Duan C, Bauchat JR, Hsieh T: Phosphatidylinositol 3-kinase is required for insulin-like growth factor-I-induced vascular smooth muscle cell proliferation and migration. *Circ Res* 86:15–23, 2000
29. Hanke JH, Gardner JP, Dow RL, *et al*: Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem* 271:695–701, 1996
30. Pricci F, Pugliese G, Romano G, *et al*: Insulin-like growth factors I and II stimulate extracellular matrix production in human glomerular mesangial cells. Comparison with transforming growth factor-beta. *Endocrinology* 137:879–885, 1996
31. Conti FG, Striker LJ, Elliot SJ, *et al*: Synthesis and release of insulin-like growth factor I by mesangial cells in culture. *Am J Physiol* 255(6 Pt. 2):F1214–F1219, 1988
32. Thomas S, Gruden G, Burt D, *et al*: Serum induces vascular endothelial growth factor production by human mesangial cells, in *Molecular and Cell Biology of Type 2 Diabetes and Its Complications,* edited by Belfiore F, Lorenzi M, Molinatti GM, *et al*, Basel, Karger, 1998, pp 221–224
33. Punglia RS, Lu M, Hsu J, *et al*: Regulation of vascular endothelial growth factor expression by insulin-like growth factor I. *Diabetes* 46:1619–1626, 1997
34. Bermont L, Lamielle F, Fauconnet S, *et al*: Regulation of vascular endothelial growth factor expression by insulin-like growth factor-I in endometrial adenocarcinoma cells. *Int J Cancer* 85:117–123, 2000
35. Abrass CK, Raugi GJ, Gabourel LS, Lovett DH: Insulin and insulin-like growth factor I binding to cultured rat glomerular mesangial cells. *Endocrinology* 123:2432–2439, 1988
36. Mukhopadhyay D, Tsiokas L, Zhou XM, *et al*: Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. *Nature* 375:577–581, 1995
37. Mazure NM, Chen EY, Laderoute KR, Giaccia AJ: Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood* 90:3322–3331, 1997
38. Goad DL, Rubin J, Wang H, *et al*: Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. *Endocrinology* 137:2262–2268, 1996
39. Miele C, Rochford JJ, Filippa N, *et al*: Insulin and insulin-like growth factor-I induce vascular endothelial growth factor mRNA expression via different signaling pathways. *J Biol Chem* 275: 21695–21702, 2000
40. Fredriksson JM, Lindquist JM, Bronnikov GE, *et al*: Norepinephrine induces vascular endothelial growth factor gene expression in brown adipocytes through a beta-adrenoreceptor/cAMP/protein kinase A pathway involving Src but independently of Erk1/2. *J Biol Chem* 275:13802–13811, 2000
41. Mukhopadhyay D, Tsiokas L, Sukhatme VP: Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. *Cancer Res* 55:6161–6165, 1995
42. Urso B, Cope DL, Kalloo-Hosein HE, *et al*: Differences in signaling properties of the cytoplasmic domains of the insulin receptor and insulin-like growth factor receptor in 3T3–L1 adipocytes. *J Biol Chem* 274:30864–30873, 1999
43. Hirschberg R, Kopple JD, Blantz RC, Tucker BJ: Effects of recombinant human insulin-like growth factor I on glomerular dynamics in the rat. *J Clin Invest* 87:1200–1206, 1991
44. Oemar BS, Foellmer HG, Hodgdon-Anandant L, Rosenzweig SA: Regulation of insulin-like growth factor I receptors in diabetic mesangial cells. *J Biol Chem* 266:2369–2373, 1991
45. Hiragushi K, Sugimoto H, Shikata K, *et al*: Nitric oxide system is involved in glomerular hyperfiltration in Japanese normo- and micro-albuminuric patients with type 2 diabetes. *Diabetes Res Clin Pract* 53:149–159, 2001
46. Veelken R, Hilgers KF, Hartner A, *et al*: Nitric oxide synthase isoforms and glomerular hyperfiltration in early diabetic nephropathy. *J Am Soc Nephrol* 11:71–79, 2000
47. De Vriese AS, Stoenoiu MS, Elger M, *et al*: Diabetes-induced microvascular dysfunction in the hydronephrotic kidney: Role of nitric oxide. *Kidney Int* 60:202–210, 2001
48. Hood JD, Meininger CJ, Ziche M, Granger HJ: VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am J Physiol* 274 (3 Pt. 2):H1054–H1058, 1998
49. Tonshoff B, Kaskel FJ, Moore LC: Effects of insulin-like growth factor I on the renal juxtamedullary microvasculature. *Am J Physiol* 274(1 Pt. 2):F120–F128, 1998
50. Tsukahara H, Gordienko DV, Tonshoff B, *et al*: Direct demonstration of insulin-like growth factor-I-induced nitric oxide production by endothelial cells. *Kidney Int* 45:598–604, 1994
51. Grant MB, Mames RN, Fitzgerald C, *et al*: Insulin-like growth factor I as an angiogenic agent. In vivo and in vitro studies. *Ann N Y Acad Sci* 692:230–242, 1993
52. Miller JW, Adamis AP, Aiello LP: Vascular endothelial growth factor in ocular neovascularization and proliferative diabetic retinopathy. *Diabetes Metab Rev* 13:37–50, 1997
53. Smith LE, Shen W, Perruzzi C, *et al*: Regulation of vascular endothelial growth factor-dependent retinal neovascularization by insulin-like growth factor-1 receptor. *Nat Med* 5:1390–1395, 1999
54. Hellstrom A, Perruzzi C, Ju M, *et al*: Low IGF-I suppresses VEGF-survival signaling in retinal endothelial cells: Direct correlation with clinical retinopathy of prematurity. *Proc Natl Acad Sci USA* 98:5804–5808, 2001